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(54) Title: AN IMMUNOLOGICAL METHOD OF TESTING CONCENTRATION

(57) Abstract

A method of quantifying one species of test antigen or test antibody in a test solution using a test strip. The test strip of porous material such as nitrocellulose paper of nylon membrane, with a species of primary antigen or antibody immobilised on it, is dipped at one end into test solution. The test solution is allowed to migrate toward the other end of the test strip. The test strips are washed and bound test antigen or test antibody is detected by a method such as colour development by an enzyme linked secondary antibody directed at the test antigen or antibody. The length of the coloured area is compared to the length of coloured area produced by a known concentration of the same species of antigen or antibody to caluclate concentration.

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AN IMMUNOLOGICAL METHOD OF TESTING CONCENTRATION

This invention relates to the quantification of molecules in solution by immunological techniques, in particular by the use of test strips.

It has been discovered that the concentration of a species of test antigen or antibody in a test solution can be determined by allowing the test solution to migrate along the length of a test strip with primary antibody or antigen able to bind the test molecule immobilised on it.

After applying a test to visualise the bound test antigen or antibody the antigen or antibody to be tested is found on a part of a strip proximal to the point at which the solution containing the antigen or antibody is loaded, and a sharp cut-off point is found beyond which no antigen or antibody is detectable.

It is further found that the distance between the cut-off point and the point at which the antigen or antibody-containing solution is applied, is directly proportional to the concentration of the antigen or antibody in the solution. Thus, this can be used as a method of determining the concentration of any antigen or antibody in a solution.

It should be noted that the present method differs in some respects from conventional immunodiffusion techniques; the most obvious being that the present method relies on capillary action to bring the antigen or antibody to be tested in juxtaposition with primary antibody or antigen rather than relying on diffusion. Another major difference is that the primary antibody or antigen is immobilised on the porous material, rather than being relatively mobile within a gel.

Thus the invention could be said to reside in a method of quantifying the concentration of one species of test immunoreactive molecules in a test solution, including the steps of:-

applying the test solution to one end of a test strip, said test strip including a sheet of porous material and a second species of immunoreactive molecules immobilised on said sheet of porous material, the said second species of immunoreactive molecules being selected so as to be able to bind to the said one species of test

immunoreactive molecules,

allowing the test solution to migrate toward an opposite end of the test strip,

substantially washing out any of the one species of test immunoreactive molecules not bound to the said second species of immunoreactive molecules, and

applying an indicator means for the visualisation of any of the said one species of test immunoreactive molecules retained on the test strip

The porous material of the test-strip may be material such as various grades of paper, nitrocellulose, or nylon membrane.

In a preferred form the visualisation means includes the steps of washing said strip to a pre-determined degree of stringency, adding a secondary antibody, with a specificity that is reactive with the first immunoreactive molecule, to said strip, said secondary antibody being bound by a detection stimulator, and applying detection means.

In one form the detection stimulator is radiolabelling of the secondary antibody. Thus on exposing photographic film to the Test strips, a record of the position of the test molecules is obtained.

In a further form the detection stimulator is an enzyme capable of producing colour change to said strip on the addition of substrate. The enzymes could be enzymes such as Horse Radish Peroxidase or Alkaline Phosphatase.

Alternatively the detection stimulator is the biotinylation of the secondary antibody.

In one form the porous material is nylon membrane and the method of coating includes the steps of agitating a sheet of nylon membrane in a solution of primary antibody, blocking any remaining unreacted sites by the addition of a concentrated solution of protein, said protein being unreactive with the visualising means. This protein could, for example, be gelatine.

In another form the invention could be said to reside in a method of quantifying the concentration of a species test antigen in a test solution comprising the steps of:-

applying the said test solution to one end of a test strip, the said test strip including a sheet of porous material that has a species of primary antibody immobilized on the said sheet of porous material, the said species of primary antibody being selected so as to be able to bind to the said species test antigen,

allowing the test solution to migrate toward an opposite end of the test strip,

substantially washing out any of the said species of antigen not bound to the species of primary antibody, and

applying an indicator means for the visualisation of the said species of test antigen retained on the test strip.

In a further form the invention could be said to reside in an arrangement for quantifying the concentration of one species of test immunoreactive molecules in a test solution including:

a test strip holder,

a test strip including a sheet of porous material that has a second species of immunoreactive molecules immobilised on said sheet of porous material,

a vessel for holding the test solution of one species of test immunoreactive molecules up to a first level, said second species of immunoreactive molecules being selected as being able to bind the said one species of test immunoreactive molecules,

said test strip holder being adapted to hold said test strip within the said vessel so that the first level defines a loading point on the test strip

said arrangement further including indicator means for visualisation of the said one species of test immunoreactive molecules retained on the test strip.

In an alternate form the invention could be said to reside in an arrangement for quantifying the concentration of first immunoreactive molecules in a solution including:

a test strip holder,

a test strip including a sheet of porous material that has second

immunoreactive molecules immobilised on it

a vessel for holding the solution of the first immunoreactiv molecules up to a first level, the said vessel being adapted for washing of uncomplexed first immunoreactive molecules from said test strip,said first immunoreactive adapted to be bound by the said first immunoreactive molecules,

said test strip holder being adapted to hold said test strip within the said vessel so that the first level defines a loading point on the test strip

said apparatus further including means for visualisation of the said first immunoreactive molecules complexed to second immunoreactive molecules and retained on the test strip.

For ease of explanation the first species of test immunoreactive molecule will now be referred to as antigen and the second species of immunoreactive molecule will be referred to as antibody or primary antibody. It is to be understood however that the first species of test immunoreactive molecule can be either antibody or an antigen, and the "primary antibody" can be either antibody or antigen.

A possible explanation of the observed phenomenon is the following: The dried, antibody-coated test strip is dipped to a specified depth and for a specified period of time into the test solution. During this time, the antigen-containing test solution is drawn up the strip by capillary diffusion and as the test antigen comes into contact with the immobilised primary antibody it is removed from the migrating solution and becomes bound, via the primary antibody, to the strip.

As more test antigen is drawn from the test solution reservoir, it must pass further up the test strip before coming into contact with uncomplexed antibody and thereby becoming bound to the strip.

If then, a high concentration of antigen is present in the solution under test, the primary antibody on the lower part of the test strip will become saturated rapidly and antigen will reach progressively higher regions of the strip before being bound by the primary antibody on the strip.

Where test antigen is present in extremely high concentrations, the

antibody on the test strip might become completely saturated and free antigens would travel with the solvent front. In this situation, after development of the assay, the entire strip would be coloured and obviously the result would not be considered quantitative.

Where test antigen is present in extremely low concentrations the antibody on the part of the strip which is dipped into the antigen-containing test solution might not become fully saturated during the time period of the assay. In this case, there would be no migration of the test antigen up the strip and following development of the assay, the coloured area which would correspond to the portion of the strip dipped in the antigen containing test solution would not be truly quantitative.

Between these two extremes over a given period of time, the height of bound antigen will be proportional to the concentration of antigen in the test solution.

There is another possible explanation of the observed phenomenon in that as the test antigen containing solution migrates along the porous strips, antigen complexes with the immobilised antibody, which is retained on the strips. As the antigen solution moves up the strip gradually the concentration decreases by virtue of the fact that some becomes bound to the antibody, and a cut-off point is reached where the antigen in the solution is exhausted. Beyond that cut-off point, no more antigen is available to bind. Thus, under this explanation, a linear relationship between distance travelled and concentration of the antigen still exists, but instead of a postulated concentration gradient forming together with an equivalence point with its associated precipitation matrix, as is found in conventional immunodiffusion tests, the cut-off point may be due to the exhaustion of antigen in a given solution.

Notwithstanding the explanation or the phenomenon, the method has many and varied applications and could be useful as a method of testing most molecules able to react with an antibody. The solvent for the antigen containing test solution is envisaged as being aqueous, although other solutions may be applicable provided that antibody

binding is not inhibited.

It is possible to make the assay accurately quantitative such that if a protocol is followed exactly, a direct read off of test antigen concentration or any given migration distance will be possible.

However, in one form the method includes the steps of comparing the distance from loading point to cut off point of an unknown immunoreactive molecule contain solution to that distance for solutions of known concentration of the immunoreactive molecule.

For a better understanding the invention will now be described with reference to specific embodiments and figures wherein;

FIG.1 is a a typical example the relationship between distance between the loading point and the cut off point on a test strip and the concentration in the test solution,

FIG. 2 is a perspective exploded view of a first embodiment of an arrangement according to the invention,

FIG. 3 is a plan view of the washing vessel from above,

FIG. 4 is a cross-sectional view from A-A of figure 3 of the washing vessel, and

FIG. 5 is a more detailed view of a clip for holding the test strips.

In a first example of a method according to the invention the test molecule is Human IgG.

The test strip is based on nylon membrane, and the preparation of the test strip is as follows:-

1. A sheet of nylon membrane (Hybond-N, Amersham International plc) is immersed in coating buffer (0.1M Sodium carbonate/ bicarbonate buffer pH 9.6) (1ml/cm^2 of test strip) and gently agitated on a rocking platform for five minutes at room temperature to ensure

complete wetting of the membrane.

2. The coating buffer is then removed and replaced with Rabbit anti-Human IgG (IgG fraction) diluted in coating buffer to give 10 $\mu g/cm^2$ and 0.5ml/cm^2. The membrane is then gently agitated as before at room temperature for two hours.

3. The membrane is then washed three times in phosphate buffered saline pH7.2 with Tween 20 (0.1%) for five minutes each wash, followed by gentle agitation in blocking buffer (3% gelatin) for one hour at room temperature.

4. Finally the membrane is washed in PBS-Tween as previously, rinsed in distiled water and then dried overnight at room temperature over silica gel.

5. 4mm strips are then cut to form the test strips.

It is to be understood that this is the method used for making test strips to test for the presence of one specific molecule, and it is to be understood that other protocols may be used to make up strips to test for the same molecule. Alternative materials may be used, and chemical linking may be necessary to immobilise some antigens on the strip.

The method of determining the concentration of the test molecule according to this second example is as follows.

The test strips are mounted into test strip holders, one end of which are immersed into a vessel containing the test solution, up to a line marked in the vessel. The test solution can be made up with Phosphate Buffered Saline pH 7.2 (PBS). The test strips are left immersed and undisturbed in the test solution containing vessel for 15 minutes to allow the test solution to migrate to the other end. The test strips are washed after removing them from the test solution by gently shaking them in a washing buffer (such as PBS-Tween 20 (0.1%) (PBS-Tw)) to remove unbound test molecules.

To indicate where the bound material is the test strips are immersed in a conjugate solution, which in this example is Anti-human IgG conjugated to Horse Radish Peroxidase and diluted in PBS-Tw with gelatin (1%). The strips are removed from the conjugate solution after 5 minutes and then washed, as before in PBS-Tw to remove unbound secondary antibody. The test strips are then immersed in enzyme substrate solution which in this specific example is 100 µg/ml 3,3'-Diaminobenzidine tetrahydrochloride, 0.2µl/ml 30%Hydrogen peroxide, in 100mM citrate buffer pH5.0. After 5 minutes the test strips are rinsed under tap water for 30 seconds and the length of the coloured zone in each strip is measured.

A graph of the relationship between length of the coloured zone and concentration of IgG in the test solution is shown in FIG. 1. Shown are the lengths of the coloured zone of five samples (in centimetres) against the concentration of IgG in the test solution. The plotted points are shown as filled in square boxes

The assay in this example has been adjusted to detect material in the range of approximately 2 - 50 µg/ml whereas the sensitivity of the test can be increased so as to detect lower levels of test molecules. A lower detection level of 100ng/ml is achievable by altering the parameters of the test strip. The lower detection level can be adjusted up or down by alteration of the concentration of the coating antibody solution. The range can also be adjusted by varying parameters such as the length of the test strip and incubation (dip) time.

Figures 2, 3, 4 and illustrate one embodiment of an arrangement for testing the concentration of one species of test immunoreactive molecule according to the invention. Test strips 1 made of nylon membrane or nitrocellulose paper are held by test strip holders which comprises plastic clips 2 that clip around the test strips and "push fit" into a holder base 3. The holder base 3 has a tapered edge to ensure a leak free fit into the washing vessel 4.

The test strip clips 2 are made of plastics material and include two hinged flaps 5 at either end of the clips 2 with a tongue and groove locking mechanism to lock the flaps into a closed position on the

holder. The test strips can be fitted into the clips 2 when the flaps are in the open position and are locked into position by snapping the hinged flaps 5 shut.

The washing vessel 4 is used for (i) holding the test solution (ii) as a washing vessel and (iii) for holding the enzyme substrate solution. The washing vessel 4 comprises a base 6 with walls 7 of the vessel extending upwardly therefrom. Two internal shoulders 8 extend longitudinally along the base, leaving there between a narrow channel. The narrow channel serves to locate the strip holders centrally and also reduces the volume of sample solution required.

Conjugate tubes 9 are provided that can be supported by a stand 10. The conjugate tubes contain freeze dried, enzyme conjugated antisera having specificity for the antigens detected by the antisera on the corresponding nylon strips. The test strips supported by the clips 2 are adapted to fit into the conjugate tubes.

It should be stressed however that in principle this assay can be used to measure any antigenic material to which an antibody can be raised. In addition the assay can be used for measuring antibody concentration by immobilising purified antigen on the strip, dipping the strip into an antibody-containing sample (e.g. blood) and developing the strip with an enzyme-labelled antigen. An example of the usage of the assay in this situation might be for measuring the immunity of a population to a particular disease. The ease of use of this assay makes it particularly applicable for use "in the field".

Whilst specific embodiments have been described in detail it is to be realised that the invention is not to be limited thereto but can include various modifications falling within the spirit and scope of the invention.

1. A method of quantifying th concentration of one species of test immunoreactive molecules in a test solution, including the steps of:-

applying the test solution to one end of a test strip, said test strip including a sheet of porous material and a second species of immunoreactive molecules immobilised on said sheet of porous material, the said second species of immunoreactive molecules being selected so as to be able to bind to the said one species of test immunoreactive molecules,

allowing the test solution to migrate toward an opposite end of the test strip,

substantially washing out any of the one species of test immunoreactive molecules not bound to the said second species of immunoreactive molecules, and

applying an indicator means for the visualisation of any of the said one species of test immunoreactive molecules retained on the test strip.

2. A method as in claim 1 wherein the said test strips are maintained in a substantially vertical position, with a lowermost end of the test strip being immersed in the test solution whilst the said test solution is allowed to migrate upwardly to the uppermost end of the said test strip.

3. A method as in claim 1 or 2 wherein the indicator means includes the steps of:-

adding a species of secondary antibody to said test strip, said secondary antibody being selected so as to be able to bind to the said one species of test immunoreactive molecules and being bound by a detection stimulator,

substantially washing out the said secondary antibody not bound to said one species of test immunoreactive molecules, and

applying detection means to detect said secondary antibody.

4. A method as in claim 3 wherein the detection stimulator is radiolabel.

5. A method as in claim 3 wherein the detection stimulating means is an enzyme being selected so as to be able to effecting a colour

change to a substrate and thereby effecting a colour change to a portion of the said test strip having bound secondary antibody.

6. A method as in claim 5 wherein the said enzyme is Horse Radish Peroxidase

7. A method as in claim 5 wherein the said enzyme is Alkaline Phosphatase.

8. A method as in claim 3 wherein the detection stimulator is the biotinylation of the secondary antibody.

9. A method as in any one of the preceding claims wherein the porous material is nitrocellulose paper.

10. A method as in any one of claims 1 to 8 wherein the porous material is nylon membrane.

11. A method of quantifying the concentration of a species test antigen in a test solution comprising the steps of:-

applying the said test solution to one end of a test strip, the said test strip including a sheet of porous material that has a species of primary antibody immobilized on the said sheet of porous material, the said species of primary antibody being selected so as to be able to bind to the said species test antigen,

allowing the test solution to migrate toward an opposite end of the test strip,

substantially washing out any of the said species of antigen not bound to the species of primary antibody, and

applying an indicator means for the visualisation of the said species of test antigen retained on the test strip.

12. A method of quantifying a species of test antibody in a test solution, comprising the steps of:-

applying the test solution to one end of a test strip, said test strip including a sheet of porous material that has a species of antigen immobilised on it, said species of antigen being selected so as to be able to be bound by the said species of test antibody,

allowing the test solution to migrate toward an opposite end of the test strip,

substantially washing out any of the said species of antibody not bound to the said species of antigen, and

applying an indicator means for the visualisation of the antibody retained on the test strip.

13. An arrangement for quantifying the concentration of one species of test immunoreactive molecules in a test solution including:

a test strip holder,

a test strip including a sheet of porous material that has a second species of immunoreactive molecules immobilised on said sheet of porous material,

a vessel for holding the test solution of one species of test immunoreactive molecules up to a first level, said second species of immunoreactive molecules being selected as being able to bind the said one species of test immunoreactive molecules,

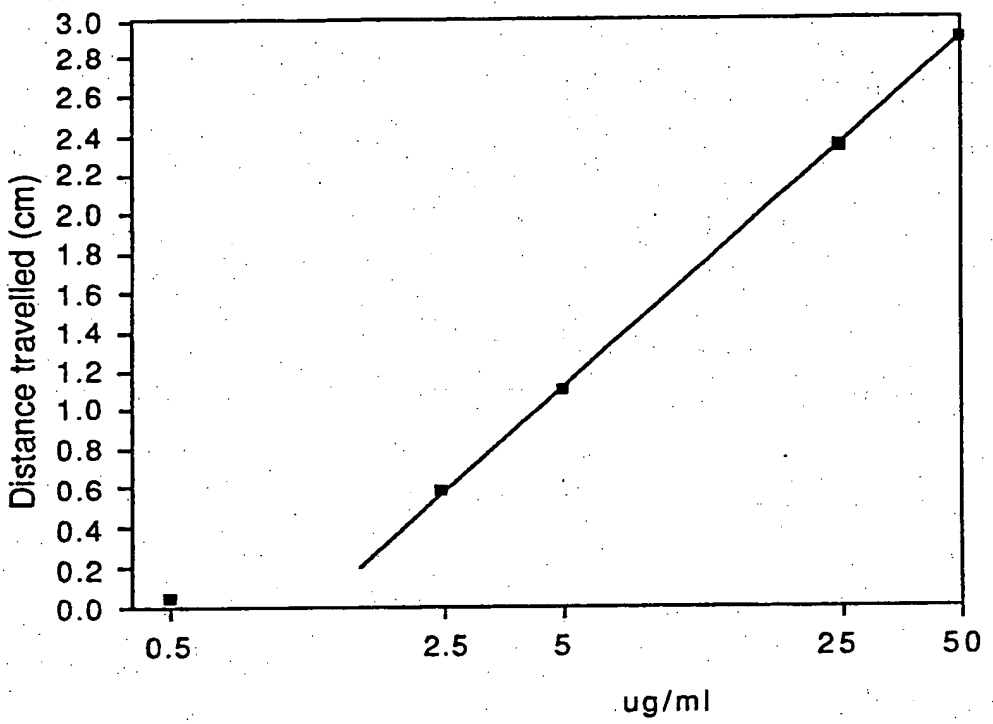
said test strip holder being adapted to hold said test strip within the said vessel so that the first level defines a loading point on the test strip

said arrangement further including indicator means for visualisation of the said one species of test immunoreactive molecules retained on the test strip.

14. An arrangement as in claim 14 wherein the said vessel is adapted for washing of any unbound molecules of the said one species of immunoreactive molecules from said test strip

15. A method of quantifying the concentration of one species of immunoreactive molecule in a test solution as hereinbefore described with reference to the examples.

16. An arrangement for quantifying the concentration of one species of immunoreactive molecule in a test solution as hereinbefore described with reference to the figures.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ug/ml

FIG 1

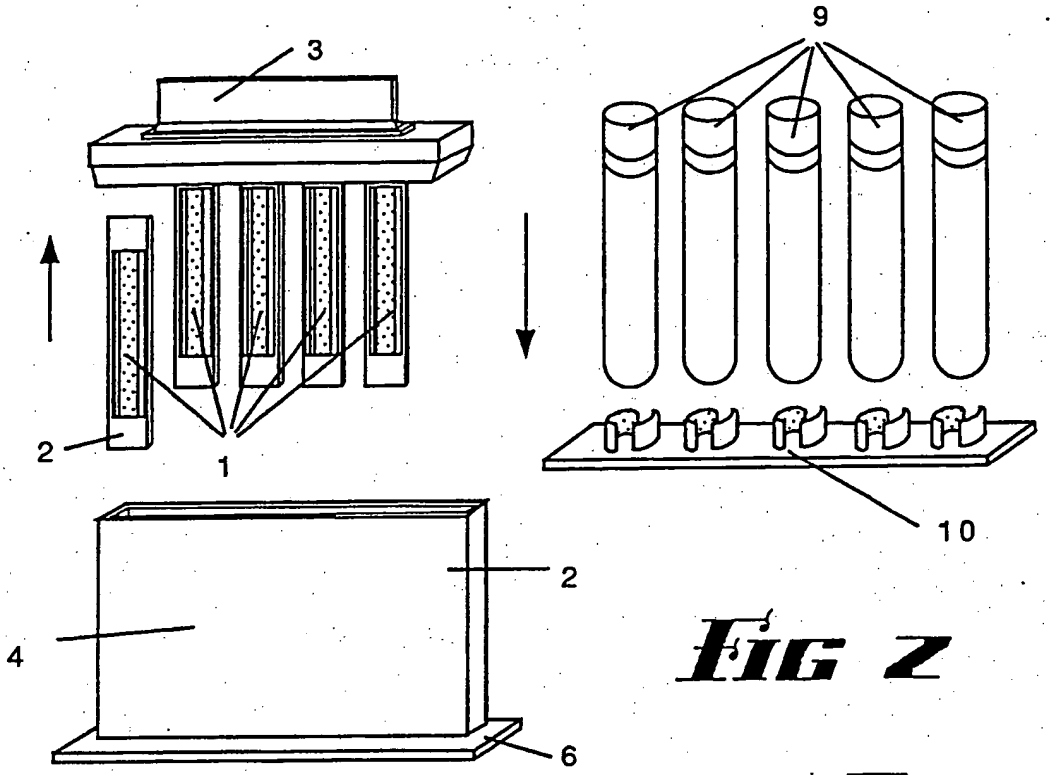

FIG 2

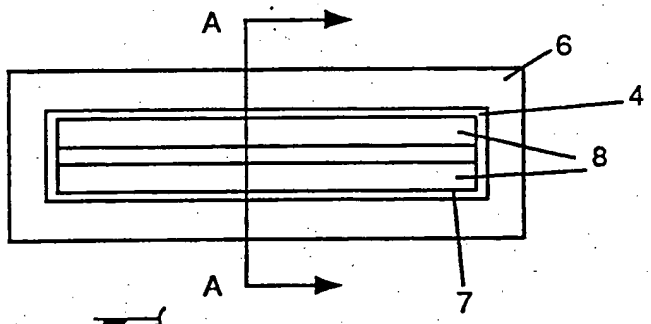

FIG 3

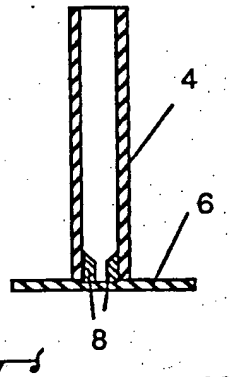

FIG 4

FIG 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00008

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] G01N 33/558

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPI and WPIL USPA Keywords: Immuno ( ) Test Strip, migrate, porous

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU : IPC as above
Chemical Abstracts; keywords: Immuno ( ) Test Strip, migrate, porous

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	AU,B 10563/76 (508642) (AB, Kabi) 28 July 1977 (28.07.77). See entire document. TOGK.	1-12, 15
X	AU,B, 16849/83 (566877) (Syva Co.) 19 January 1984 (19.01.84). See pages 1-5.	1-12, 15
X	EP,A 0246643 (DNA Plant Technology Operating Co.) 25 November 1987 (25.11.87)	1-15
X	AU,A 53624/86 (Syntex, Inc.) 28 July 1987 (28.07.87). See entire document, particularly pages 1-5	1-15

(continued)

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 April 1990 (12.04.90)	30 April 1990
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	A.W. BESTOW

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	AU,A 14305/88 (Syntex, Inc.) 13 October 1988 (13.10.88). See pages 1-5	1-15
X	AU,A 13595/88 (Beaton, Dickinson and Company) 29 September 1988 (29.9.88)	1-15

V. [] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[] Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[] The additional search fees were accompanied by applicant's protest.

[] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 90/00008

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
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	JP 59090056	NL 7600128	NO 760233
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	JP 1063865		

END OF ANNEX